Report as of FY2010 for 2010PA141B: "Coupled Analytical and Biological Analyses of Endocrine Disrupting Compounds (EDCs) of Emerging Concern in Municipal Wastewater Sources in Philadelphia"

Publications

- Articles in Refereed Scientific Journals:
  - Johnson, M. Candice., Mohan P. Achary and Rominder P. Suri (in preparation). Estimating the Relative Estrogenic Potential using a Combined Experiment Approach . Environmental Toxicology and Chemistry Journal, submitted.

Report Follows

PROJECT TITLE & PRINCIPAL INVESTIGATORS

Coupled Analytical and Biological Analysis of Endocrine Disrupting Compounds (EDCs) of Emerging Concern in Municipal Wastewater Sources in Philadelphia

Principal Investigator: Mohan P. Achary, PhD, Assistant Professor, Department of Radiation Oncology, Temple University School of Medicine, Philadelphia; email: achary@temple.edu; Phone: 215 707 8185

Co-Principal Investigator: Rominder Suri, PhD, PE, Associate Professor, Department of Civil and Environmental Engineering, Temple University, Philadelphia; email: rominder.suri@temple.edu; Phone: 215 204 2378

PROBLEM & RESEARCH OBJECTIVES

Endocrine disrupting activity has been detected in wastewater effluents of several water treatment facilities; thereby, conferring estrogenicity to receiving waters [1]. Evidence suggests that these hormones result in undesired characteristics to aquatic life even in the low ng/L range [2]. This is particularly because of the vast number of estrogens (of both natural and synthetic origin) that are combined in the complex wastewater matrix. Sophisticated analytical techniques allow the determination of the micro-constituents which may exist in the water. However, the biological activity of the wastewater matrix is best determined through the use of bioassays. Additionally, through the combined use of bioassays and analytical techniques any possible interaction amongst the components can be identified and discerned.

Proposed objectives:

> A. Determination of the concentrations of estrogens present in the influent and effluent samples of 3 WWTPs of Philadelphia using LC/MS/MS and LC Q-TOF-Mass Spectrometry
> B. Effect directed analyses of wastewater derived estrogens using the E-Screen and the Yeast Estrogen Screen

To achieve the objectives of this study we focused on the following specific aims:

1) Determining the concentrations of target estrogens in the wastewater sample.
2) Determining whether or not an interaction occurs amongst the hormones in the wastewater matrix.
3) Predicting the biological activity of the influent and effluent samples based on chemical data.

METHODOLOGY

The techniques used include the Yeast Estrogen Screen (YES) assay and the E-Screen assay which utilizes estrogen receptor positive MCF-7 cells as representative bioassays for the determination of estrogenicity, and Liquid chromatography tandem mass spectroscopy (LC-MS/MS) for the quantification of hormones present in the samples.

LC-MS/MS

These instruments offer superb sensitivity and resolution for analyte detection. Estrogen hormones are extracted from the wastewater samples using Solid Phase Extraction (SPE). This allowed for the detection of analytes that are present in the ng/L range by concentration of the sample. We followed the methods that are already established for the detection of estrogen hormones in the analytical laboratory of Dr. Suri, the Co-PI in this project.

Water Collection and Estrogen Extraction

Grab wastewater influent and effluent samples were collected from a local municipal wastewater treatment works facility and stored in amber glass bottles. The facility serves 9 municipalities, 2 hospitals, and receives 60% of its water from municipal and 40% industrial sources. Secondary treatment is achieved using the activated sludge process. Within 24 hours after water collection samples were vacuum filtered through a 0.45µm membrane in preparation for solid phase extraction (SPE) of the estrogens. SPE was performed on a C-18 cartridge (Varian BondElute) under gentle vacuum. Samples were eluted in 6 mL of methanol after pre-cleaning with 6mL of 30% methanol in water. This served to reduce matrix interferences which may hinder analytical detection. Eluents were dried under vacuum and reconstituted in a 50% methanol/water solution for detection on UPLC-MS/MS. Influent and effluent samples were enriched 500 and 750 times for analytical detection and 4900 and 7400 times respectively for biological assays. In preparation for biological analysis, dried eluents were reconstituted in pure ethanol.

Yeast Estrogen Screen (YES) Assay

The YES was carried out as per the Routledge and Sumpter method [3]. This assay makes use of a modified strain of yeast developed by Routledge and Sumpter and provided by Dr. Joseph Colosi of De Sales University. In order to construct this recombinant strain, the human cDNA sequence coding for the ER (hER) was integrated into the genome of the yeast *Saccharomyces cerevisiae*, under the control of a yeast promoter. The yeast also contained a plasmid carrying an ERE-LacZ construct controlling the oestrogen-induced expression of the reporter gene *LacZ,* encoding the enzyme b-galactosidase. Thus, in the presence of estrogen the yeast synthesizes b-galactosidase, which splits the yellow chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG), present in the assay-medium, into galactose and the chromophore chlorophenol red, yielding a dark red compound. The b-galactosidase activity is quantified spectrophotometrically at 540nm [3].

Stock solutions of 17β-Estradiol, Estriol, 17α-Dihydroequilin, and Estrone were prepared in ethanol and stored at -20C. Mixtures of the above compounds were prepared by transferring a relevant amount of a single solution onto a flat bottom 96 well plate and allowing the ethanol to evaporate to dryness before adding the other mixture constituents. Assay medium (comprising of yeast at a final optical density (OD) of 0.1, CPRG, and growth medium) were then added to the well. The plate was then incubated for 72 hours at 32^{o}C. The conversion of chlorophenol red-β-D-galactopyranoside (CPRG) to chlorophenol red (CPR) was measured by recording absorbance values at 540nm and correcting for yeast growth at 630nm. Similarly, 5-10µL of the extracted samples were placed in the plates and allowed to dry prior to the addition of assay medium. The relative estrogenic potential (REP) of the extracts was used as a measure of estrogenicity. In case of the extracts the biological response is plotted against the relative enrichment factor (REF)

which represents the final concentration factor if the extract to which the biological organism was exposed. The REF is calculated as [4]:

$REF = \left(\frac{V_{estrogens}}{V_{extract}} \right) \bullet \left(\frac{V_{bioassay}^{extract}}{V_{bioassay}} \right)$; where V represents the volume of the respective samples.

Without statistical evidence of parallelism amongst the dose response curves, multipoint estimates of the REP were determined at the EC10, EC20, EC50, and EC80 levels when possible. Percent reductions in estrogenicity were then calculated at each effect level then averaged to yield a single estimate in reductions.

E-Screen (ES) Assay

The E-Screen utilizes the proliferative ability of estrogen positive MCF-7 breast cancer cells [5]. The MCF-7-BUS cells used in our study were received from the laboratory of Dr. Ana Soto of Tufts University. These cells were used as they are sensitive and can produce a response that is approximately six times than that of original MCF-7 cell line. The assay is based on the fact that MCF-7 cells naturally express the estrogen receptor isoforms and undergo increased growth rates as a result of the activation of the estrogen receptor. The experimental conditions involve seeding cells under estrogen free conditions; that is, in growth medium without phenol red and with Charcoal-dextran stripped fetal bovine serum. The stripping of the serum removes any compounds which may inhibit the response to estrogens.

The cells were routinely maintained in DMEM supplemented with 2% HEPES, 2% Glutamine, 1% Penn/strep, and 10% FBS. Experimental medium contained phenol-red free DMEM supplemented with HEPES, Glutamine, and Penn/strep as above in addition to 10% charcoal stripped FBS. Stock solutions were prepared in ethanol and diluted in experimental medium such that the final ethanol concentration did not exceed 0.1%. Cells were seeded into 6-well plates at an initial density of 50,000 cells / well and allowed to attach. After 24 hours, medium containing the test compounds was added and cells incubated for 6 days. Following incubation, the cells were trypsinized and counted using a TC Biorad automatic cell counter. The estrogenic activity was quantified as the relative proliferative effect (RPE). This is simply the ratio of the highest cell yield obtained with the test substance and estradiol, and is calculated as

$$RPE = 100 \times \frac{PE_{test} - 1}{PE_{estradiol} - 1}$$

Modeling biological activity

Initial studies focused on determining the interactive potential of the hormones found at wastewater treatment when combined. The biological activity of the mixtures was analyzed using the YES. The concentration addition (CA) model was also used to investigate the additivity of specified mixtures [6-8]. In brief, the CA assumes that each mixture constituent acts as a dilution of the other, with the relative estrogenic potential (REP) of the compound serving as the dilution factor [9]. Deviations from additivity are then easily discerned as variation of the observed biological responses to the predicted outcome. The model is derived and utilized as follows;

For a binary mixture an interaction can be defined by Equation 1 [6, 10,11].

$$\frac{C_1}{EC_{X,C_1}} + \frac{C_2}{EC_{X,C_2}} = I \qquad (1)$$

where 'I' represents the interactive index, C_i is the concentration of compound C in the mixture exerting an EC_x effect and EC_{xi} represents the equal effect concentration of C alone.

If compounds 1 and 2 act additively then 'I' is equal to unity. An interaction is then defined as I assuming a value greater than 1 for synergistic activity or less than 1 for antagonism. The equation can be rearranged in terms of the total mixture concentration, C_T, and the proportion of each mixture constituent, P_x. Thus, given a fixed ratio design the following is true if additivity is assumed.

$$C_T\left[\frac{P_1}{EC_{X,C_1}} + \frac{P_2}{EC_{X,C_2}}\right] = 1, \qquad (2)$$

Equation 2 allows for the prediction of the mixture response based on the proportion of the mixture components and the standalone response of each individual component. Rearrangement gives the working formula, equation 3.

$$C_T = \left[\Sigma \frac{P_i}{EC_{X,Ci}}\right]^{-1} \qquad (3)$$

PRINCIPAL FINDINGS AND SIGNIFICANCE

LC-MS/MS determination of estrogens in wastewater

Table 1 shows the concentrations of natural estrogens found in wastewater treatment plant influent and effluent determined from the calibration curves shown in Figure 1. It appeared that 17β-estradiol was significantly removed in the treatment process whereas estriol and 17α-dihydroequilin was removed to a lesser extent, 24 and 54% respectively. Estrone on the other hand appeared to increase in the wastewater effluent by approximately 77%. This result agrees with previously published reports of metabolic conversion of 17β-estradiol to estrone in the activated sludge process [12-14]. As such, estrone is often viewed as one of the hormones primarily responsible for endocrine disruption in aquatic species [15].

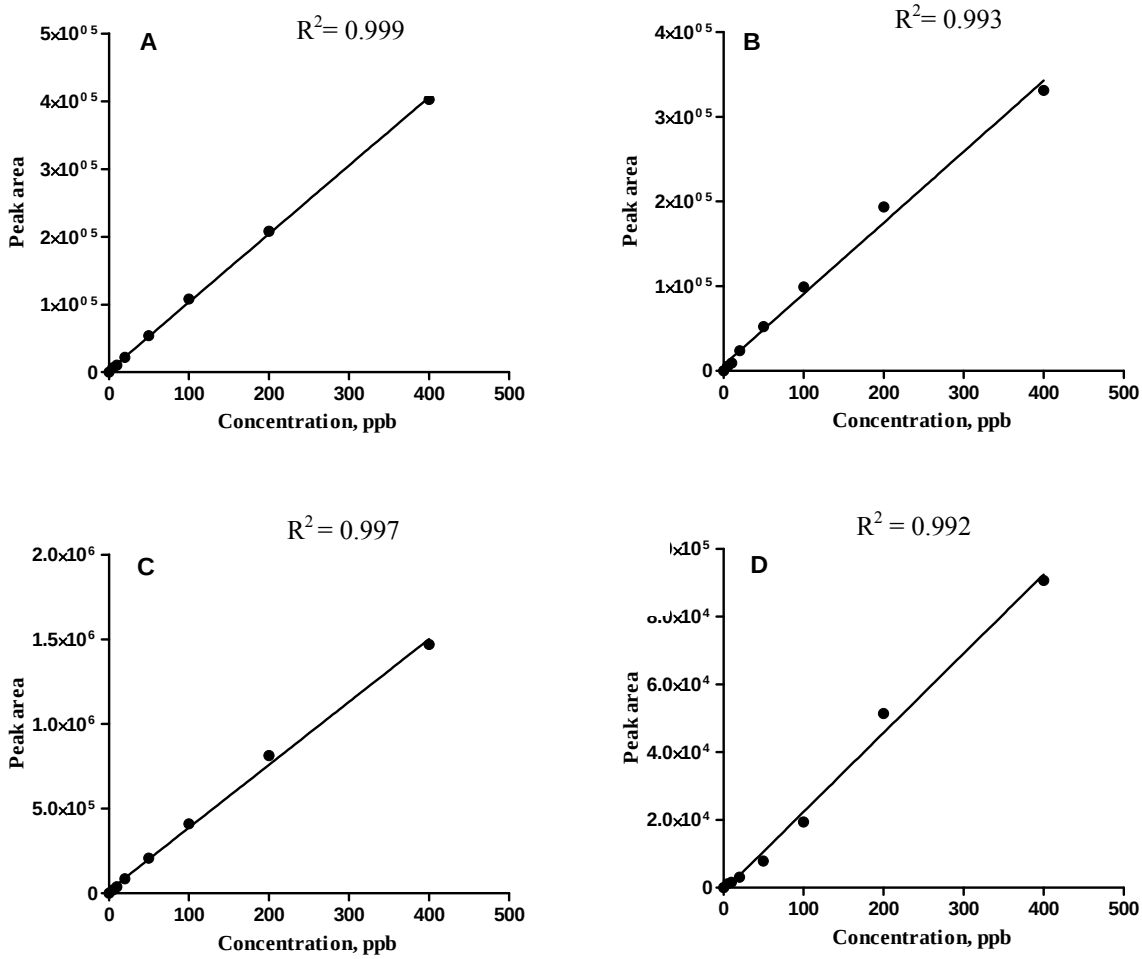


Figure 1- Calibration curves for A) estriol, B) 17β-estradiol, C) estrone, D) 17α-dihydroequilin after SPE and determined using LC-MS/MS

Table 1- Concentration of estrogen hormones in the influent and effluent of the water treatment facility

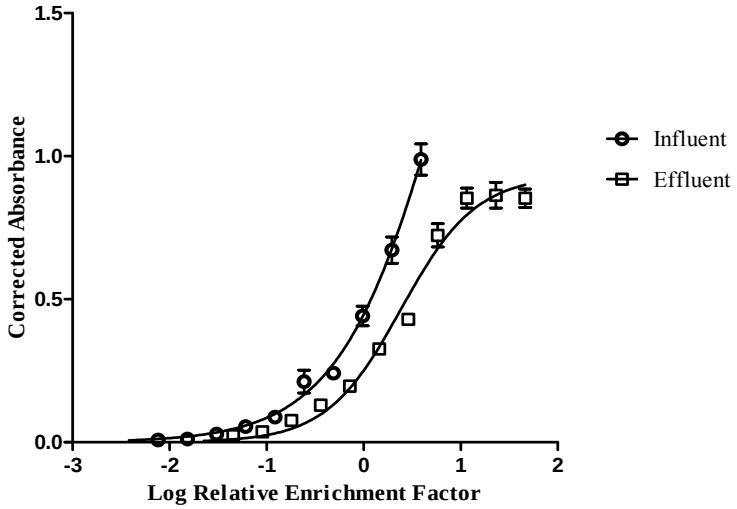
	Estriol	17β-estradiol	estrone	17α-dihydroequilin
Influent (ng/L)	8.66	5.07	0.15	679.18
Effluent (ng/L)	6.55	*ND	0.65	311.61
% Reductions	24.35	100	**N/A	54.12

* Not detected

** An increase in final effluent concentration is observed

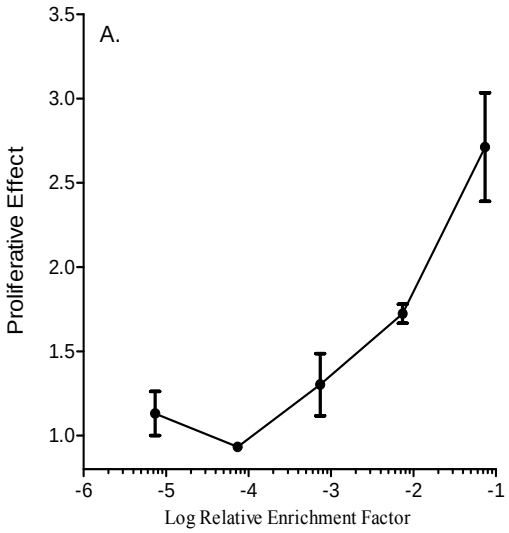
Biological activity of influent and effluent assessed using YES and E-screen

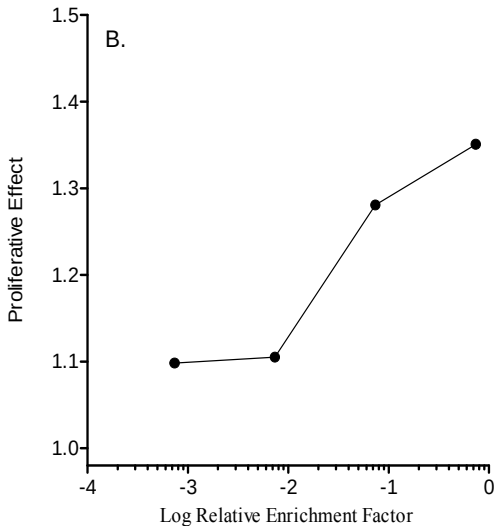
As expected from the chemical data above, the wastewater effluent contained significant residual activity in spite of treatment. In order to quantify the changes in the estrogenic activity observed without confirmation of parallelism to the standard 17β-estradiol, the relative estrogenic potential (REP) was calculated at several effect levels and averaged. The residual activity of the effluent was 61% less than that of the influent reflecting the incomplete degradation of the hormones investigated (Figure 2). Similar reductions in the proliferative effect of the influent and effluent samples were observed with the E-screen assay which showed approximately 79.5% reduction in estrogenic activity. The effluent samples also yielded a submaximal response in the E-screen assay and a full dose response curve was not achieved in the influent samples due to the toxic effect, Figure 3.



Effect Level (%)	Influent REP	Effluent REP	% Reduction
80	0.0206	N/D	N/D
50	0.0193	0.00751	61.1
20	0.0307	0.0131	57.5
10	0.0467	0.0164	64.8
		Average	61.1

Figure 2- Dose response curves and percent reductions in the estrogenicity of influent and effluent samples





	Influent	*Effluent	% Reduction in RPE
Relative proliferative effect (RPE)	39.8	8.16	79.5

*Pending replication of these experiments

Figure 3- A. influent, and B. effluent sample responses in the E-screen assay. The highest concentration tested in both samples was toxic to the cells as determined by microscopic observation.

Comparison of chemical and biological estradiol equivalents in YES

The chemically determined concentrations were converted into expected biological responses using the CA model described above. The predictions were made based on the single compound dose response curves shown in figure 4. Laboratory prepared mixtures of 17β-estradiol, estriol, estrone, and 17α-dihydroequilin showed that there was no expected interaction amongst the hormones as the response could be predicted within the 95% confidence interval of the observed response, figure 5. In the case of the wastewater samples, the predicted enrichment factors were calculated through the division of the total mixture concentration determined by the model by the summed concentration determined by LC-MS/MS. The CA model roughly predicted the responses up to about the EC_{10} in both cases; thereafter, deviations from additivity were observed. This appears to arise as a result of dissimilar hill slopes in the case of both the influent and effluent samples. The submaximal effluent curve was also not predicted by the model. Since an interaction is not expected based on a simulation of the mixture composition in ethanol, it is hypothesized that this deviation may result from either the effect of unknown components such as humic acids, or a decrease in enzyme activity associated with the wastewater matrix. Of interest however, is the fact that the reduction in estrogenicity determined by the chemical analysis (61.5%) is in good agreement with that determined by the bioassay.

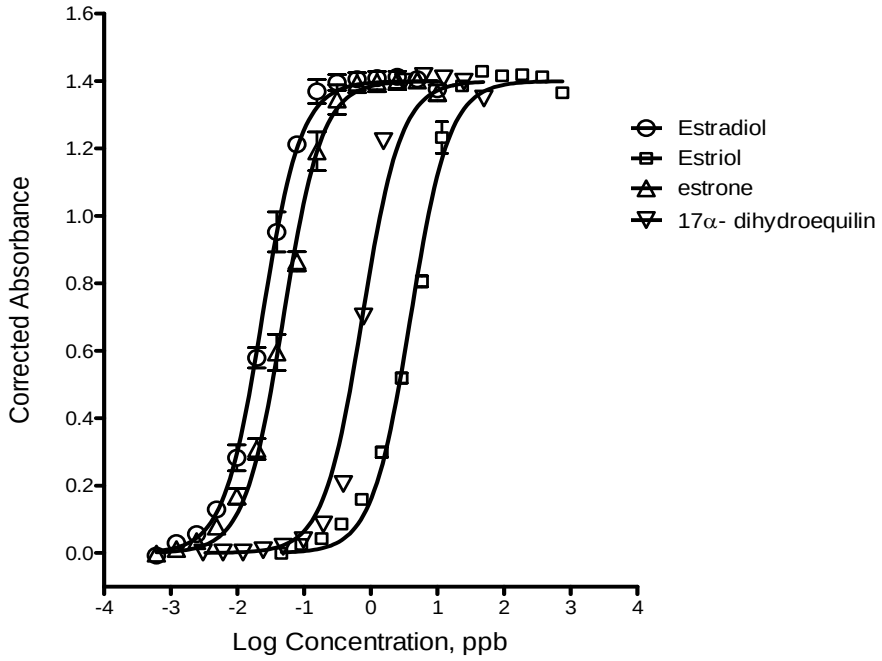


Figure 4- Single compound analysis in the YES assay.

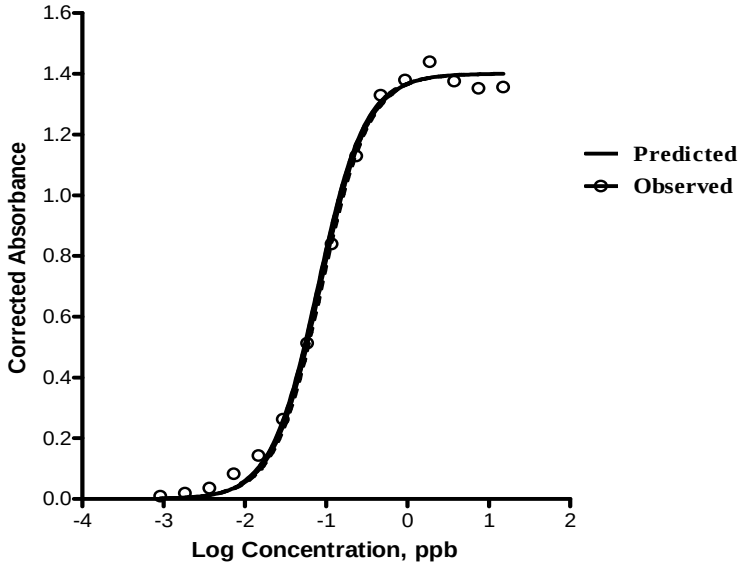


Figure 5. 17β-estradiol, estriol, estrone, and 17α-dihydroequilin were combined and the CA model used to predict the responses. The observed responses were well predicted by the model indicating that there is no potential for an interaction amongst these hormones.

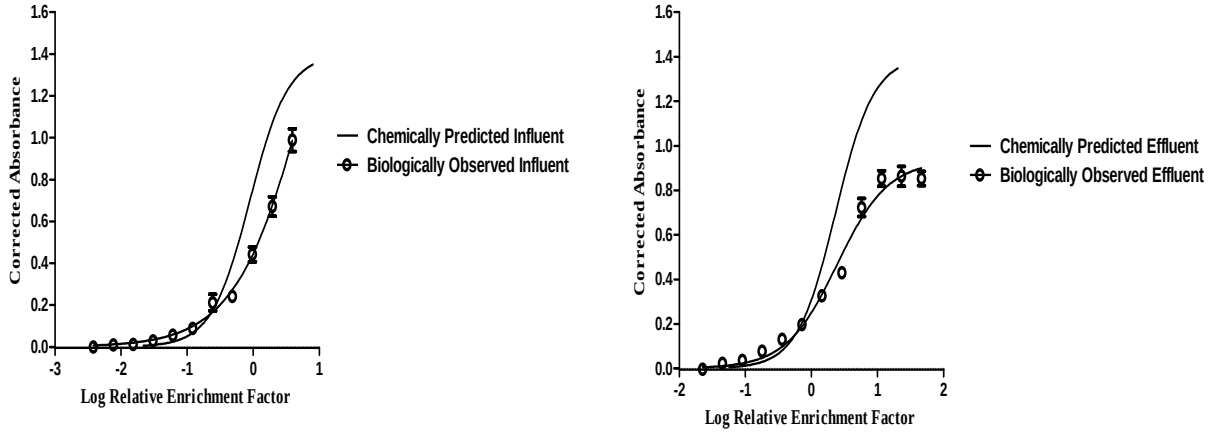


Figure 6. Calculated and observed dose response curves for all effect levels of the influent and effluent samples in the yeast estrogen screen (YES) assay. An antagonistic-like activity was observed in both the wastewater influent and effluent samples.

Future studies will include testing recoveries in wastewater matrix by spiking with a labeled internal standard and assessment of additional treatment facilities during both winter and summer seasons to assess the variability in the degradation patterns which may occur.

STUDENTS & POSTDOCS SUPPORTED (name, major, degree)

1. Ms. Candice Johnson, MS: Doctoral student
2. Mr. Benjamin Conway, BS: Graduate student

Both Ms. Johnson and Mr. Conway assisted Dr. Achary in achieving the results provided in this report.

Note: For additional information please visit, www.hdcsetac.org

References cited:

1. Cargouet, M.; Perdiz, D.; Mouatassim-Souali, A.; Tamisier-Karolak, S.; Levi, Y., Assessment of river contamination by estrogenic compounds in Paris area (France). *Science of the Total Environment* **2004,** *324*, (1-3), 55-66.
2. Routledge, E. J.; Sheahan, D.; Desbrow, C.; Brighty, G. C.; Waldock, M.; Sumpter, J. P., Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environmental Science & Technology* **1998,** *32*, (11), 1559-1565.

3. Routledge, E. J.; Sumpter, J. P., Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry* **1996,** *15*, (3), 241-248.
4. Macova, M; B.I. Escher, B.I.; Reungoat, J; Carswell, S; Lee Chue, K; Keller, J; and Mueller, J.F., Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. *Water Research* **2010**, 44, (2), 477-492.
5. Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernandez, M. F.; Olea, N.; Serrano, F. O., The E-Screen assay as a tool to identify estrogens - an update on estrogenic environemntal pollutants. *Environmental Health Perspectives* **1995,** *103*, 113-122.
6. Payne, J.; Rajapakse, N.; Wilkins, M.; Kortenkamp, A., Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environmental Health Perspectives* **2000,** *108*, (10), 983-987.
7. Rajapakse, N.; Ong, D.; Kortenkamp, A., Defining the impact of weakly estrogenic chemicals on the action of steroidal estrogens. *Toxicological Sciences* **2001,** *60*, (2), 296-304.
8. Rajapakse, N.; Silva, E.; Kortenkamp, A., Combining xenoestrogens at levels below individual No-observed-effect concentrations dramatically enhances steroid hormone action. *Environmental Health Perspectives* **2002,** *110*, (9), 917-921.
9. Tallarida, R. J.; Stone, D. J.; Raffa, R. B., Efficient designs for studying synergistic drug combinations. *Life Sciences* **1997,** *61*, (26), PL417-PL425.
10. Loewe, S., The problem of synergism and anatagonism of combined drugs. *Arzneimittel-Forschung-Drug Research* **1953,** *3*, (6), 285-290.
11. Tallarida, R. J., *Drug synergism and dose-effect data analysis*. Chapman & Hall/CRC Press: Boca Raton, 2000; p viii, 247 p.
12. Servos, M. R.; Bennie, D. T.; Burnison, B. K.; Jurkovic, A.; McInnis, R.; Neheli, T.; Schnell, A.; Seto, P.; Smyth, S. A.; Ternes, T. A., Distribution of estrogens, 17 beta-estradiol and estrone, in Canadian municipal wastewater treatment plants. *Science of the Total Environment* **2005,** *336*, (1-3), 155-170.
13. Baronti, C.; Curini, R.; D'Ascenzo, G.; Di Corcia, A.; Gentili, A.; Samperi, R., Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environmental Science & Technology* **2000,** *34*, (24), 5059-5066.
14. Yu, C. P.; Roh, H.; Chu, K. H., 17 beta-estradiol-degrading bacteria isolated from activated sludge. *Environmental Science & Technology* **2007,** *41*, (2), 486-492.
15. D'Ascenzo, G.; Di Corcia, A.; Gentili, A.; Mancini, R.; Mastropasqua, R.; Nazzari, M.; Samperi, R., Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. *Science of the Total Environment* **2003,** *302*, (1-3), 199-209.